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(57) Abstract

The invention is based on the discovery that biological and non–biological materials can be sterilized, decontaminated, or disinfected by repeatedly cycling between relatively high and low pressures. Pressure cycling can be carried out at low, ambient, or elevated temperatures (e.g., from about –20 °C to about 95 °C). New methods based on this discovery can have applications in, for example, the preparation of vaccines, the sterilization of blood plasma or serum, the decontamination of military devices, and the disinfection of medical equipment. The new methods can also be incorporated into production processes or research procedures.

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RAPID CRYOBARIC STERILIZATION AND VACCINE PREPARATION

Cross Reference To Related Application

This application claims priority from U.S. Serial Number 09/097,852, filed June 15, 1998, and from U.S. Serial Number 09/165,829, filed October 2, 1998, both of which are incorporated herein by reference in their entirety.

Background of the Invention

The invention relates to methods for sterilizing materials and preparing vaccines.

Various methods and devices exist for the sterilization, decontamination, or disinfection of biological and non-biological materials. These methods include thermal destruction (e.g., burning), heat sterilization, irradiation (e.g., ultraviolet or ionizing irradiation), gas sterilization (e.g., using ethylene oxide), photosensitization, membrane sterilization, and the use of chemical disinfectants (e.g., formaldehyde, glutaraldehyde, alcohols, mercury compounds, quaternary ammonium compounds, halogenated compounds, solvent/detergent systems, or peroxides).

Heat sterilization (e.g., autoclaving) is often used, for example, for sterilizing medical solutions prior to use in a patient. Heat sterilization typically requires heating a solution to 121°C for a minimum of 15 minutes under pressure in an autoclave, maintaining the heat and pressure conditions for a period of time sufficient to kill bacteria, fungi, and protists and inactivate viruses in the solution.

Many reusable medical articles and materials are not suitable for disinfection or sterilization in an autoclave. For example, plastic parts on medical devices, hemodialyzers, and fiber optic devices are commonly

sterilized by chemical germicide treatment. In general, germicides require up to several hours of treatment for the inactivation of microorganisms.

To ensure sterility in pharmaceutical production, gas sterilization is often employed. However, gas sterilization (e.g., using ethylene oxide) can be time-consuming, requiring prehumidification, heating, and evacuation of a sample chamber, followed by treatment with high concentrations of the gas for up to 20 hours at a time.

When properly used, traditional disinfectants can inactivate vegetative bacteria, certain fungi, and lipophilic or medium-sized viruses. However, these disinfectants often do not arrest tubercle bacillus, spore-forming bacteria, or non-lipophilic or small-sized viruses.

Another method for lysing cells, and thereby sterilizing a sample is described in *Microbiology* (Davis et al., Harper & Row, Hagerstown, MD, 1980). This procedure of freezing and thawing the sample is believed to exert its effect through formation of tiny pockets of ice within the cells when a suspension of bacteria is frozen. The ice crystals and the high localized concentrations of salts both cause damage to the bacteria. A single freezing event is generally sufficient to kill only some of the bacteria, but repeated freeze-thaw cycles result in a progressive decrease in viability. Lethality is correlated with slow freezing and rapid thawing.

Traditional freeze-thaw methods are limited in the speed of the freeze-thaw cycle by the time needed to transfer heat to and from the center of the sample to effect phase changes. The equilibrium rate is particularly slow in the case of large volume samples (e.g., about 100 ml or larger). Sterilization efficiency of the traditional methods is limited by the impracticality of performing a large number of freeze-thaw cycles by those methods.

Traditional methods of food preservation include pasteurization, in which a food is held at an elevated temperature for a period of time.

There is an ongoing need to develop improved methods for sterilization, particularly for the inactivation of viruses and other microbes in materials that also contain proteins having activity that one desires to retain, such as clotting factors, antibodies, blood enzymes (e.g., lipases, phosphatases), and growth factors. The development of methods for inactivation of non-encapsulated viruses is especially challenging, since the outer coats of such viruses generally include proteins similar to the proteins one wishes to retain.

Summary of the Invention

The invention is based on the discovery that biological and non-biological materials can be sterilized, decontaminated, or disinfected by repeatedly cycling between relatively high and low pressures. Pressure cycling can be carried out at low, ambient, or elevated temperatures (e.g., from about -40°C to about 95°C). New methods based on this discovery can have applications in, for example, the preparation of vaccines, the sterilization of blood plasma or serum, the decontamination of military devices, food and beverage production, and the disinfection of medical equipment. The new methods can also be incorporated into production processes or research procedures.

In general, the invention features a method for sterilizing a material. The method includes the steps of providing a material at an initial pressure (e.g., 1 atm) and temperature (e.g., 25°C, lower temperatures such as 0°C, -5°C, -25°C, -40°C or lower); increasing the pressure to an elevated pressure sufficient to inactivate at least some (e.g., at least about 10%, 25%, 50%, 75%, 90%, 95%, 99%, or even substantially all) microbes contained in the material (e.g., in the range of about 5,000 psi to about 95,000 psi,

or in the range of about 10,000 psi to about 75,000 psi, or in the range of about 95,000 psi to about 150,000); and subsequently decreasing the pressure to a reduced pressure, which may be about the same as, less than, or greater than the initial pressure (e.g., around 1 atm), to provide a sterilized material (i.e., a material having a reduced titer of microbes).

In some cases, the material includes a protein. In such cases, it may be desirable that the elevated pressure be insufficient to irreversibly denature proteins during the time that the pressure is at an elevated level. One skilled in the art would understand that "denaturing a protein" means denaturing a sufficient amount of the protein so as to reduce or destroy the usefulness of the protein for a particular application. Typically, irreversibly denaturing more than about 50% of the protein molecules in the sample would render the sample an unsatisfactory source of the protein. In some cases, however, retention of even 10% or less of the protein activity is adequate.

The material can be chilled to a subzero temperature (e.g., from about -40°C to about 0°C, especially between about -20° and about -5) either before or after the pressure is increased. The temperature can be subsequently increased, either before or after the pressure is decreased.

The pressure can optionally be repeatedly cycled (e.g., 2, 3, 5, 10, or even 100 or more times) between the elevated pressure and the initial pressure. Such cycling can be carried out at the initial temperature, at a low temperature (e.g., subzero temperatures such as between -40°C and 0°C, or between -20°C and -5°C), or while the material is being cooled to a low temperature. The timing of the cycles may be such that the temperature of the material is allowed to equilibrate (e.g., to the temperature of the walls of a reaction vessel in which the method is carried out) prior to each cycle.

In some cases, a material at low temperature can be in the solid (i.e., frozen) state at the initial pressure, but in the liquid (i.e., molten, or thawed) state at the elevated pressure. In such cases, pressure cycling causes concomitant freeze-thaw cycling. The temporal pattern of pulsation can, optionally, be altered. During each cycle, the pressure is alternately raised and then lowered. The ratio of the time at high pressure to the time at low pressure is termed as the "pulsation pattern ratio." A pulsation pattern ratio greater than 1:1 (e.g., 2:1 or more) can give optimal inactivation of contaminants in most cases, whereas a pulsation ratio less than 1:1 can give greater retention of properly folded, sensitive proteins.

The material being sterilized can be, for example, a biological sample, blood plasma, therapeutic and/or diagnostic products derived from blood plasma, biological fluids, medical fluids, medicaments, research solutions and reagents, serum, living tissue, medical or military equipment, a foodstuff, a pharmaceutical preparation, or a vaccine. The material being sterilized can be initially infected with, contaminated with, or otherwise contain, for example, one or more of a bacterium, a virus, a fungus, a protist, a spore former, a protozoan parasites, malaria-inducing organisms, giardia, or virally infected cells.

The invention also features a method for inactivating a virus in a material. The method includes the steps of providing a material at an initial pressure and temperature; and exposing the material to repeated pressure cycles (e.g., 2 to 100 cycles, more than about 3, 10, 50, 100, 1,000, or 10,000 cycles). Each pressure cycle includes the steps of increasing the pressure to an elevated pressure (e.g., between about 10,000 psi and about 120,000 psi, between about 40,000 psi and about 100,000 psi, or between about 70,000 psi and about 90,000 psi), maintaining an elevated pressure for a time period t_e, decreasing the pressure to a reduced pressure, and maintaining the material at a reduced

pressure (e.g., a pressure less than the elevated pressure, and less than, equal to, or greater than the initial pressure), for a time period t_i. The elevated pressure is sufficient such that each cycle inactivates at least some (e.g., at least about 1%, 5%, 10%, 25%, 50% or more) of the virus in the material when the elevated pressure is maintained for time t_e (e.g., between about 0.5 and about 300 seconds, or between about 10 and about 30 seconds).

In some cases, the material includes a protein. In such cases, the elevated pressure can be a pressure that would be sufficient to irreversibly denature substantially all (e.g., 50%, 75%, 90%, 95% or more) of the protein if the elevated pressure were maintained for a time substantially longer than t_e (e.g., 2, 3, 5, 10, or 100 times t_e, or more), but is insufficient to irreversibly denature the protein when the elevated pressure is maintained for only time period t_e or less.

The protein can be, for example, a blood clotting factor (e.g., factor VII, VIII, IX, XI, and or XIII), an immunoglobulin (e.g., IgG, IgM), a monomeric protein, a multimeric protein, or a mixture of proteins.

The virus can be an encapsulated or non-encapsulated virus (e.g., human parvovirus B19, porcine parvovirus, bovine parvovirus, human immunodeficiency virus, herpes simplex virus, hepatitis A virus, bacteriophage MS2, or transfusion transmitted virus).

The method can also include the step of cooling the material to a temperature T_e, (e.g., about -40°C to about 10°C, or about -25°C to about -10°C), prior to exposing the material to the elevated pressure.

Any of the new methods described above can also be used to produce vaccines against specific microbes. For example, a suspension of microbial cells can be obtained, sterilized by one of the new methods (e.g., the method that involves pressure cycling, and potentially freeze/thaw cycling, at a subzero temperature), and combined with an adjuvant to

produce a vaccine. If there are toxins present in the suspension, these can removed (e.g., after the sterilization step).

In some cases, it can be useful to include an additive such as a phase-change catalyst (e.g., glass particles), a protein-stabilizing agent (e.g., a sugar, glycerol, a hydrophilic polymer, a cyclodextrin, a caprylate, acetyl tryptophanoate, polyethylene glycol, anti-oxidant, or a protein specific ligand), or a nucleic-acid binding compound (e.g., a photosensitizer such as a psoralen) in conjunction with the material to be sterilized. Some such additives can subsequently be removed by centrifugation or filtration, if necessary.

Materials sterilized by any of the above methods are also considered to be an aspect of the invention.

In another embodiment, the invention features an apparatus for sterilizing a material. The apparatus includes a pressurization vessel adapted to transmit an external pressure to a material within itself. The vessel needs to be capable of withstanding an elevated pressure (e.g., pressures encountered in the practice of any of the new methods described above), must be capable of fitting in a pressure cycling apparatus (e.g., such as those described in PCT US97/03232), and may include a valve that allows aseptic recovery of the sterilized material. In some cases, the apparatus can also include heating and cooling devices (e.g., a heater and a refrigerator).

In still another embodiment, the invention features a method for sterilizing a material that includes proteins. The method includes the step of providing the material at an initial pressure; rapidly increasing the pressure to a pressure sufficient to inactivate microbes; and quickly restoring the initial pressure to provide a sterilized material and to avoid substantial aggregation of proteins. The elevated pressure may be sufficient to denature most proteins (e.g., between about 65,000 psi and about 85,000

psi); due to the short duration of the pressure cycles (e.g., between about 1 s and about 300 s), and in some cases the low temperatures (e.g., between about -10°C and about -40°C), however, the new methods avoid causing excessive irreversible denaturation of the proteins in the materials sterilized thereby.

In yet another embodiment, the invention features another method for sterilizing a material that includes proteins. The method includes the steps of providing the material at an initial pressure; adding one or more protein-stabilizing reagents (e.g., sugars such as glucose; glycerol; a hydrophilic polymer; a cyclodextrin; a caprylate; acetyl tryptophanoate; polyethylene glycol; an anti-oxidant; or a protein specific ligand) to the material; increasing the pressure to an enhanced pressure (e.g., about 10,000 to 70,000-80,000 psi, depending on the stability of the protein); incubating the material for a time sufficient for sterilization to occur without substantial loss of protein function; and restoring the pressure to the initial pressure, to provide a sterilized material. Again, the pressure may be repeatedly cycled.

In any of the above methods, the material to be sterilized can be provided in its final packaging, the packaging being able to transmit pressure without rupture. For example, the packaging can be hermetically sealed in flexible plastic (e.g., PVC or polyethylene). Alternatively, the packaging can be a syringe and the pressure can be transmitted via a plunger.

The invention also features a method for pressurizing an infectious material. The method includes the steps of charging the material into a container adapted to transmit an external pressure to the material; submerging the container in a sterilizing chemical solution (e.g., containing an oxidizing agent, an alcohol, urea, a guanidinium salt, an acid, or a base); and pressurizing the material within the container.

In any of the above methods, the pH of the material to be sterilized can optionally be adjusted to greater than about 10 (e.g., between 10 and 14, or between 11 and 12) or less than about 4 (e.g., between 0 and 4, or between 2 and 3), prior to increasing pressure. Such pH adjustment can be useful, for example, for inactivation of pH-sensitive microbes (e.g., parvovirus) or microbes in materials that include proteins that are resistant to extremes of pH (e.g., IgG), can accelerate the sterilization methods, and can, in some cases, allow the methods to be carried out at lower pressures overall.

As used herein, the term "subzero temperature" means a temperature lower than 0°C (e.g., -1°C, -5°C, -10°C, -20°C, or lower). All temperatures herein are in degrees Celsius unless otherwise stated, and are simply denoted by "°C". Units of pressure herein are expressed in pounds per square inch (psi) or in atmospheres (atm). 1 atmosphere is about 14.5 psi, 1 bar, or 101.3 kilopascals.

A "cryobaric process" is a process that involves at least one pressure change carried out at a subzero temperature. In some cryobaric processes, the pressure is cycled between two pressures (e.g., about 14.5 psi to about 5,000 psi, 35,000 psi, 70,000 psi, 80,000 psi, 100,000 psi, or higher) while the temperature is either maintained at a subzero temperature or varied within a subzero temperature range.

The terms "sterilize", "disinfect", "inactivate", and "decontaminate" are used interchangeably herein, unless otherwise demanded by the context. It should be noted that "sterilization" (killing of all organisms) may not be synonymous in certain operations with "decontamination" when the contaminant is non-living, such as a protein or prion.

The new methods provide several advantages. For example, the methods can be carried out at subzero temperatures (e.g., between about -40°C and 0°C). Pressure cycling carried out at subzero temperatures can

advantageously induce oscillation between different phases of water within or outside the cells or vesicles of biological contaminants. The transition between the liquid and solid states can create physical stress on membranes, walls, and vesicles, thereby facilitating the intended processes. The range of subzero temperatures generally used in the new methods is easily accessed with relatively inexpensive equipment (e.g., commercial chilling devices) that is readily available in a range of shapes and sizes to fit a specific need. Similarly, the range of pressures required for the standard operation of the methods (e.g., from about 14.5 psi to about 30,000 psi, 70,000 psi, 80,000 psi, 100,000 psi, or higher) can be generated by devices as described in PCT US97/03232.

An apparatus for sterilization of a material by a cryobaric process will generally include a chamber for containing the material, the chamber being capable of operation at a selected elevated pressure, and a system for controlling, altering, or regulating the temperature and pressure within the chamber. The apparatus can allow for increasing and decreasing the pressure at a rapid rate sufficient to avoid denaturation of proteins during the pressurization and depressurization steps of the pressure cycles. Optimal rapidity is dependent, for example, on the temperature used in the process; generally, higher rates are required at higher temperature. The apparatus will also provide systems for removing a sterilized material in an aseptic manner from the chamber. Additionally, a typical sterilization apparatus for use with the new methods can include a variety of controls, regulators, and temperature, and/or pressure sensors. The pressurizing medium can be, for example, a water/ethylene glycol solution or other non-freezing solution or a solid such as powdered talc.

The devices necessary for carrying out the new methods can be easily adapted to conform the requirements of particular applications. For example, a small, portable

device can be obtained, thereby allowing sterilization or decontamination procedures to be carried out in the field (e.g., by paramedics or military medical personnel).

Variation of temperature can also aid lysis of microbes. Temperature, pressure, cycle count, or cycle frequency can be varied to maximize the effectiveness of the sterilization processes.

The new methods are very rapid. For example, the pressure can be cycled at a frequency of about 1 mHz to about 10 Hz, typically allowing the entire sterilization process to be completed within minutes.

The new methods allow pathogenic organisms in a material to be neutralized without concomitant denaturation of proteins. The new methods can avoid denaturation, which often occurs upon sterilization of biological materials.

Rapid and economical sterilization is achievable with a minimum of protein destruction or denaturation. The new methods can thus be advantageously used for the production of highly active vaccines. These vaccines can be superior to vaccines produced at higher temperatures, since high temperatures can cause disruption of both covalent and noncovalent bonds in proteins, and can lead to a greater degree of irreversible denaturation than the methods claimed here. High temperatures may also lead to covalent linkage of proteins to small molecules such as oxidized glucose. Another advantage of the invention is that it can be used to inactivate viruses and bacteria in patient samples prior to analysis. Laboratory workers can be subject to risk of acquiring infection (e.g., from viruses such as human immunodeficiency virus and hepatitis B virus) when handling samples of tissues, cells or fluids taken from patients for analysis. To avoid this risk, such samples can be collected into a container that has been designed to insert into a pressure cycling apparatus. A suitable container might be compressible, or might have a piston or plunger to transmit pressure. The methods of the invention can then be carried

out on the sample in the container, without exposure of the sample prior to pathogen inactivation. In one example, blood collected in a syringe is capped and the capped syringe is placed inside a pressure chamber filled with a sterilizing pressure-transmitting medium such as 70% ethanol. The sample is then treated with a method of the invention and removed for analysis.

Other advantages of the new methods include the avoidance or reduction of the need for addition of chemical additives to blood fractions; scalability of the process from single units to large, pooled samples or to continuous, on-line processes; and elimination of side effects of thermal inactivation processes on protein components.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description

The invention provides a method by which a material can be sterilized or decontaminated by high pressure in the range of about 5,000 to 95,000 psi, preferably in the range of 30,000 to 75,000 psi. The material is adjusted, either before or after pressurization, to a particular temperature

which is both compatible with preserving the desirable properties of the material, and which also allows destruction of the contaminants.

Although the temperature, pressure, number and duration of cycles, and relative timing of pressure and temperature changes can vary, the new methods are in general carried out according to the following procedure: A material is provided at initial pressure (e.g., atmospheric pressure, 14.5 psi) and temperature (e.g. ambient temperature, 25°C, or a refrigerated temperature such as 0°C to 4°C, or a frozen temperature such as -80°C to -20°C). The material is then pressurized to an elevated pressure. The pressure can then be cycled repeatedly between the elevated pressure and ambient pressure. The material can be produced in a frozen state after the final depressurization, or can be warmed to 0°C or higher before depressurization to produce a non-frozen, sterilized product.

The above processes can also be used in conjunction with methods in which a known static pressure and/or a particular pressure is maintained for a given time. In some cases, the pressure may be maintained at a greatly elevated level for an amount of time sufficient for microbe inactivation but insufficient for irreversible protein denaturation.

If a phase change is involved in any sterilization process, a catalyst may be added to accelerate the change. For example, the presence of finely-divided glass or other materials can provide nucleation sites for freezing of a sample or material.

General

Applications of the new methods include the sterilization of materials such as blood plasma from donors (e.g., for use in transfusions), the sterilization of purified or partially purified therapeutic and diagnostic proteins derived from blood, medical samples (blood, urine, fecal, hair, biopsy, or other tissue samples),

pharmaceuticals (e.g., biosynthetic antisense drugs), biopharmaceuticals, and transgenically produced proteins. The new methods can be used to arrest the proliferation of viruses in incubated materials or to sterilize such materials. The new methods can also be used to ensure sterility of cosmetics, pharmaceutics, and industrial products.

Examples of microbes that can be inactivated by the new methods include both hydrophilic and lipophilic viruses; nearly any bacteria, including, for example, *Staphylococci, Micrococci, Pyogenic streptococci*, diphtheroids (e.g., lipophilic, non-lipophilic, anaerobic diphtheroids such as *Propiobacterium*), gram-negative enteric bacilli (e.g., *Escherichia, Enterobacter, Klebsiella, Proteus, Serratia*), *Neisseria*, aerobic spore formers, mycobacteria; fungi, including, for example, yeast, *Pityrosporum ovale, Pityrosporum orbiculare, Candida albicans, Candida parapsilosis, Torulopsis glabarata*, and filamentous dermatophytic species; protists and lower multicellular organisms, including protozoan parasites; and helminth parasites; malaria-inducing organisms; giardia; and virally infected cells.

Examples of viruses that can be inactivated by the new methods include both DNA and RNA viruses, such as human parvovirus B19 ("B19"), porcine parvovirus ("PPV"), bacteriophage MS2 ("MS2"), and hepatitis A virus (HAV). Both B19 and HAV are small (about 15-30 nm), do not possess an outer envelope composed of lipids (non-enveloped), are resistant to heat and chemical treatment, and are difficult to remove by nanofiltration. PPV and MS2 are similarly resistant viruses that can serve as research models for the human pathogens B19 and HAV. Enveloped viruses (e.g., human immunodeficiency virus, hepatitis B virus, and hepatitis C virus) are also potential targets of the new methods. Currently uncharacterized viruses, such as some newly-

recognized forms of hepatitis virus and transfusion transmitted virus can also be vulnerable to the new methods.

Plasma pools often contain hepatitis C virus (HCV). Procedures for producing blood products can thus benefit from a process that inactivates HCV and other viruses. Human parvovirus B19 (B19), is another common contaminant of plasma. Hepatitis A virus (HAV) contaminants are less common, but still troublesome. Both B19 and HAV are small (about 15-30 nm), do not possess an outer envelope composed of lipids (non-enveloped), are resistant to heat and chemical treatment, and are difficult to remove by nanofiltration. Enveloped viruses (e.g., HIV, HBV, HCV) are also potential targets of the new methods. Currently uncharacterized viruses, such as some newly-recognized forms of hepatitis virus and transfusion transmitted virus (TTV) can also be vulnerable to the new methods. In addition, prion-based infectious agents such as transmissible spongiform encephalopathies are difficult to screen and to inactivate. However, because the methods of the invention can, under suitable conditions, induce protein unfolding, it may be possible to inactivate such agents by the methods of the invention.

Due to the possibility that disrupted virus particles can re-assemble after pressure treatment, it can be desirable to irreversibly degrade the nucleic acids contained in the virus. Moderately high pressures (e.g. 20,000 psi to 60,000 psi) can disrupt complexes of nucleases and their endogenous inhibitors. The activated nucleases can serve to degrade nucleic acids and thereby enhance irreversible inactivation of viruses. Additionally, moderate pressure can accelerate the activity of uninhibited enzymes. The process may be enhanced by the addition of nucleases. It is desirable in some cases to add a magnesium independent nuclease, as in the treatment of citrated plasma, since the citrate can bind magnesium ions thus inhibiting magnesium-dependent nucleases.

Alternatively, much higher pressures (e.g. 50,000 psi to 150,000 psi) can be used for sterilization of materials that are pressure-stable, such as small molecule pharmaceuticals or thermostable proteins. A pressure-cycling freeze-thaw sterilization method (e.g., a method that takes advantage of the cyclic formation of high pressure ice such as ice III, ice IV, ice V or ice VI) may also be used.

When the biological contaminants are relatively pressure stable and the material contains labile proteins that need to be retained, a variation of this method can be used. In this variation, the pressure is increased rapidly (e.g., in less than 5 seconds, or less than 1 second) to a very high maximum pressure (e.g., 100,000 psi or higher), and held at high pressure only briefly (e.g., less than 5 seconds). The pressure and time are chosen to provide a high degree of microbe inactivation, but the time is brief enough that proteins denatured by the elevated pressure conditions do not have time to aggregate into irreversible complexes to a sufficient extent before they refold into their native forms. The pressure is then rapidly released (e.g., in less than 5 seconds, or less than 1 second). The inactivation of microbes such as viruses can proceed at a much greater rate than the irreversible aggregation or denaturation of protein molecules.

Carrying out the pressure cycling at low temperature can further improve retention of active protein, by slowing the rate of aggregation and increasing the rate of protein refolding. Under certain conditions of high pressure and low temperature (e.g., 100,000 psi and -20°C), high pressure ice (i.e. ice V or ice VI) can form. Proteins that are trapped in the lattice structure of the high pressure ice are less likely to aggregate. The high pressure ice takes a finite amount of time to melt, this time being sufficient for the proteins in the material to refold while trapped in

the solid phase. The addition of glucose can also increase the rate of protein folding.

Pressure has also been shown to increase the activity of numerous enzymes. For example, RNase activity is accelerated by elevated hydrostatic pressure. This effect can be exploited in conjunction with the new methods for the inactivation of viruses. RNA viruses are readily degraded following high pressure treatment.

Blood transfusion

The new methods can be used to improve the safety of blood transfusions. Plasma protein products such as intravenous immunoglobulin ("IVIg"), factors VII, VIII, IX, XI, and XIII, albumin, von Willebrand's factor, fibrinogen, antithrombin III, protein C, C1-inhibitor, alpha-1 antitrypsin, and fibrin sealant are needed, for example, by hemophiliacs, cancer patients, and kidney dialysis patients. However, viruses present in blood products can present a risk for patients in need of those products. Even using new filtration techniques that eliminate many cells, certain bacteria and viruses can remain in the products.

Ordinarily, blood plasma is isolated by obtaining a blood sample (e.g., collected in a tube containing an anticoagulant), centrifuging the sample in a plasma separation tube, and decanting the plasma from the precipitate in the tube. Although this method frees the plasma from the bulk of the cells, some cells inevitably remain in the plasma. If the remaining cells include, for example, bacteria or viruses, diseases can be spread by transfusion. The new methods can be carried out on the plasma obtained from the above decanting method. The contaminants that remain in the plasma can be inactivated by the new methods.

Sterilization of Reagents and Media

The new methods can also be used to sterilize industrial products. For example, bovine serum is often used in molecular biology laboratories for cell cultures.

Microbial contamination of the source stock material from the supplier occurs infrequently; when it does happen, however, the economic costs and time delays can often be significant. Current methods for sterilization of fetal calf serum (e.g., heat or filtration) can inactivate functionally important proteins (e.g., growth hormones) and also cause variability from lot to lot. Moreover, even if the source stock material is initially sterile, it can become inadvertently contaminated upon opening in the laboratory. The new methods can be used in either a production process (e.g., batch or continuous) or used in individual laboratories for pretreatment of serum or other media prior to initiation of an experiment.

Vaccine Production

Vaccines are typically prepared by subjecting a solution of cultured viruses to an inactivating treatment (e.g., heating, or addition and removal of chemical denaturants).

A successful vaccine preparation method should ideally result in a high degree of viral inactivation, but should allow the product to retain its ability to stimulate a protective immune response in the patient. High pressure procedures are well suited to meet the criteria needed for successful vaccine production: since cold, pressure-denatured proteins retain a more native-like structure than do heated or chemically-denatured proteins, pressure inactivated viruses can thus be more immunogenic. Pressure-denatured proteins are also less likely to aggregate, thereby providing higher yields of vaccine. The pressure-inactivation methods described herein can be economical on a large scale since there are generally no chemicals to add or remove and, unlike heat, pressure can be transmitted rapidly through a large sample.

The specific conditions necessary for vaccine production can vary depending on the particular microbe

to be inactivated. For example, in the case of spore forming organisms, an optional pretreatment with low pressure and moderate temperature (e.g. 10,000 psi and 40°C) can be applied to cause the spores to germinate. The germinated spores can then be inactivated by the methods of the invention.

Pressure enhanced photosensitization of nucleic acids:

A method of sterilization has been described, wherein the product to be sterilized is mixed with a chemical agent that can preferentially bind to DNA or RNA and react with the nucleic acid (Radosevich, "Seminars in Thrombosis and Hemostasis," Vol. 24, No. 2, pp. 157-161, 1998). In some cases, light is used to activate the chemical moiety. Disadvantages of such a method can include collateral damage to the desired molecular components of the product to be sterilized (e.g., via non-specific reaction with chemicals or irradiation, or by imperfect or slow penetration of the inactivating chemical to the interior of the microbe). The application of elevated pressures can substantially overcome these problems by permeabilizing cells and viruses to allow entry of the inactivating chemicals. Elevated pressures can also enhance the affinity and selectivity of the molecules for the nucleic acids, thereby allowing the use of lower chemical concentrations or lower amounts of irradiation. Thus, a faster, less expensive, and more efficient method is obtained.

An apparatus for the execution of the photochemical method can include a high pressure flow-through system such as described in PCT Appln. US96/03232, having a reaction chamber that includes at least one pressure-resistant window which can be made of a material such as quartz or sapphire, and a device for irradiation of the material through that window. The flow of liquid is such that all of the material passes through the irradiated area. The material can then be collected aseptically.

The material can be introduced into the reaction chamber at high pressure or at low pressure, and then pressurized prior to irradiating.

Chemical Inactivation of Viruses

A variety of chemicals (e.g. iodine, ethyleneimine, ascorbic acid, thiophosphamide, congo red, paraformaldehyde) can be used to sterilize solutions containing labile proteins. Use of such chemicals can have negative effects, however, including slow inactivation, potential for protein damage, or the inability of compounds to penetrate to the interior of the microbe. Elevated pressure can enhance the sterilization activity of these chemicals without exacerbating the negative effects (e.g., by increasing the chemicals' effectiveness against heat stable non-encapsulated viruses such as parvovirus and hepatitis A virus.

Stabilization of proteins and enzymes

In some cases, it may be desirable to sterilize a solution or other material containing an unstable protein that would be irreversibly denatured at the pressure necessary for the sterilization procedures described above. In these cases, a stabilizing agent (e.g., amino acids such as amino acids, such as glycine, or specific ligands of proteins in the mixture, ligands of proteins to be recovered, or sugars such as glycerol, xylose, or glucose) can be added to the material prior to pressurization. For example, the human serum albumin stabilizers caprylate and acetyl tryptophanoate can be added to blood plasma samples, and the plasma samples can then be subjected to the cryobaric sterilization process without excessive destabilization of specific plasma proteins. The stabilizer can then be removed by standard methods (e.g. dialysis, filtration, chromatography).

Pressure treatment of infectious materials

Hydrostatic or pulsating pressure can be a useful tool for sterilization, cell and virus disruption, and nuclease inactivation for materials that may potentially contain agents of infectious disease. Moreover, general safety considerations call for the prevention of infection of the persons handling the material and the avoidance of contamination of other materials. One way to prevent such contamination is to use a sterilizing solution (e.g., 10% CLOROX® bleach; 70% ethanol; concentrated urea; or a guanidinium salt) or an oxidizing agent as a pressurizing medium.

For example, the material can be placed inside an enclosed and flexible container, which can then be immersed in the chemical sterilizing solution. The solution can then be sealed inside of a second, chemically inert container (i.e., to keep it from contacting the metal parts on the inside of a pressurization chamber). An inert pressurizing medium can then be used to fill the volume between the inside of pressurization chamber and the container holding the material and sterilizing solution. The container that holds the sterilizing solution can be, for example, a plastic bag, a screw top plastic container, a capped syringe, or a shrink wrapping.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

Examples

Example 1: Cryobaric Deactivation of Lambda Phage

For each of 4 samples, 2.5 μl Lambda phage stock (5 x 10^{11} pfu/ml) was diluted 100-fold by addition of about 248 μl calf serum. A fifth 2.5 μl lambda phage stock sample was diluted 100 fold in suspension medium. Two phage-serum samples (250 μl) were frozen by immersing

tubes in an ethanol-dry ice bath; three other samples were placed on ice.

One of the frozen phage-serum samples was cycled between 36,000 psi (30 seconds) and 14.7 psi (30 seconds) for 5 minutes. During pressure treatment, the temperature of this sample was maintained at -10°C. The remaining frozen sample was left at 14.7 psi throughout the experiment as a control. When this experiment was repeated, a dry-ice sample was warmed to -10°C for 5 minutes. A third phage-serum sample was pressurized to 36,000 psi for 10 minutes at 23°C. The two remaining samples, used as positive controls, were a phage-serum sample incubated at 23°C for 10 minutes with no pressure treatment and a phage-suspension medium (i.e., 0.0496 M sodium chloride, 4.06 mM magnesium sulfate, 50 mM Tris-Cl pH 7.5, and 1.0 g/l gelatin) with neither pressure nor temperature treatment, respectively. After pressure treatment, 10^2, 10^4, and 10^6 fold dilutions of phage samples were made in Suspension medium.

A culture of ampicillin resistant *E. coli* was grown to saturation in Lambda Broth (i.e., 10 g/l tryptone, 0.042 M sodium chloride), 0.2% maltose, and 10 mM magnesium sulfate (Current Protocols in Molecular Biology, page 1.11.1).

To induce infection, 100 µl of the phage sample was added to 300 µl of the *E. coli* culture prepared above, and the culture was incubated for 10 minutes at 37°C. 2.5 ml Lambda Top Agar (i.e., 10.0 g/l tryptone, 42 mM sodium chloride, 7 g/l agar) was added to each phage-*E. coli* mixture, vortexed, and immediately spread onto Lambda plates (i.e., 10 g/l tryptone, 0.042 M sodium chloride, 10.0 g/l agar in 90 mm petri dishes).

After incubation of the plates for 16 hours at 37°C, lambda plaques were either counted, or, for plates with high confluence, the total surface area covered by plaques was estimated. The plaque forming units per

milliliter (pfu/ml) were calculated by multiplying the dilution factor by the number of plaques appearing on each plate.

The plaque forming activity of Lambda phage was reduced by 5 orders of magnitude by alternating hydrostatic pressure at -10°C. The density of the frozen, pressure-cycled sample was found to be 9.4×10^5 pfu/ml, while that of the frozen, unpressurized control was 3.2×10^{11} pfu/ml. In a second experiment, the plaque forming activity was reduced from 9×10^{10} pfu/ml to 6.4×10^4 pfu/ml after alternating pressure treatment. In contrast to the frozen samples, there was only a 3-fold decrease in plaque forming activity in the room temperature samples. The sample held at 36,000 psi for 5 minutes at 23°C yielded 1.2×10^{11} pfu, and the control maintained at 14.7 psi had 3.1×10^{11} pfu.

Serum had very little if any effect on plaque forming activity. There were 50×10^{11} pfu in the sample diluted in Suspension medium, and the sample diluted 100-fold into serum decreased only to 3.1×10^{11} pfu. Freezing just once at atmospheric pressure (14.7 psi) apparently had no effect on plaque forming activity. There were 3.2×10^{11} pfu in the frozen sample and 3.1×10^{11} pfu in the sample incubated at room temperature (23°C).

Example 2: Effect of pulsation frequency on viral inactivation.

An experiment was carried out to determine the effect of pulsation frequency on the inactivation of lambda bacteriophage in serum.

A serum sample was inoculated with the virus and treated as in Example 1, but with varying frequencies of pulsation and a maximum pressure of 40,000 psi. All samples were treated at -6°C, the total time of treatment for all samples was 15 minutes, and the time spent at

high and low pressures was 7.5 minutes in each experiment. The experiment was carried out twice for each set of experimental conditions. The viral titers were measured as in Example 1.

A parallel experiment was carried out on a model of a therapeutic protein to see if its activity was maintained. Anti fluorescein goat IgG (Chemicon International; Tecuma, CA) was prepared to a concentration of 4 mg/ml in 5% glucose and 0.3% NaCl. The solution was then subjected to the same treatment as in the bacteriophage inactivation experiment and assayed by measurement of the ability to quench the fluorescence of a 50 nM solution of fluorescein. The following data were obtained:

Frequency (cycles per hour)	phage titer (trial #1)	Trial #2	% IgG activity
0	5.0×10^8	1.1×10^7	100
4	4.6×10^4	9.9×10^2	94
12	3.0×10^3	7.7×10^2	97
20	3.4×10^2	5.0×10^1	92
40	4.8×10^2	0	95
60	7.0×10^1	0	100

This experiment demonstrates that the pulsation of pressure at low temperature can have a significant effect on the rate of viral inactivation, and can be useful in the production of therapeutic and diagnostic blood products. The process can operate under conditions of pressure and temperature that are consistent with high recovery of properly folded therapeutic proteins and can be effective against many types of viruses.

Example 3: Acceleration of Nuclease Activity in a Hyperbaric Sterilization Process

Adult bovine serum is diluted to 50% (v/v) with water and chilled to 0°C. Four aliquots of the serum are

dispensed into 250 μl microcentrifuge tubes such that there is 25 μl of air in each tube. The tubes are kept on ice until use. 2 μg of pUC19 plasmid DNA and 2 μg of yeast total RNA (Sigma) in 5 μl of 50 mM Tris buffer, pH 8.0, are added to each sample at the appropriate time. 5 μl of water is added to sample #4 before pressurization. The reactions are stopped by adding of 10 mM vanadyl ribonucleoside complexes and placing the samples on ice. The treatment of these samples are as follows:

Sample #1: control sample (i.e., nucleic acids only). In this sample, the vanadylribonucleosides are added prior to the nucleic acids.

Sample #2: serum and nucleic acid mixture, incubated at 25°C for 10 minutes. The reaction is immediately stopped as described above.

Sample #3: serum and nucleic acid mixture, pressurized to 60,000 psi at 25°C for 10 minutes. The reaction is stopped as described above.

Sample #4: serum, pressurized to 60,000 psi at 25°C for 10 minutes; the nucleic acids are then added, the mixture is incubated for 10 minutes, and the reaction is stopped as described above.

All samples are extracted with an equal volume of phenol/chloroform/isoamyl alcohol mixture to remove proteins, followed by precipitation of the nucleic acids in 70% ethanol. The nucleic acid pellet is re-dissolved in 20 μl of TE buffer. 10 μl of the resulting solution is loaded on a 0.8% agarose gel for electrophoresis. After electrophoresis, DNA is visualized by ethidium bromide fluorescence. 5 μl of the remaining sample is quantified using PicoGreen and Ribogreen dyes (Molecular Probes).

Samples #3 and #4 both show increased degradation with respect to sample #2. The nucleic acids in sample #4 are more degraded than the nucleic acids in sample #3. These results demonstrate that pressure can accelerate

blood nucleases both by directly stimulating activity and by releasing inhibitors.

Example 4: Pressure- and Temperature-Induced Inactivation of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae was grown in YPD liquid medium (1% yeast extract, 2% peptone, 2% dextrose) until the cultures reached a density of 2 x 10^6 cells/ml. The *S. cerevisiae* samples were then diluted 1:10 in calf serum and then subjected to various pressures and temperatures.

First, the temperature was held constant at 23.8°C during a pressurization process. One sample was maintained at 36,000 psi for 10 minutes at 23.8°C. A second sample was cycled between 36,000 psi and 14.7 psi at 30 second intervals for 10 minutes at 23.8°C. The pressure and temperature were held constant at 14.7 psi and 23.8°C, respectively, for a third sample as a positive control.

For the next three samples, temperature was reduced while equivalent pressure treatments were carried out. Thus, a fourth sample was cooled to -3.6°C and then pressurized to 36,000 psi for 10 minutes. A fifth sample was also cooled to -3.6°C, then subjected to 10 cycles of pressure alternating between 36,000 psi and 14.7 psi at 30 sec intervals. As a positive control, a sixth sample was cooled to -3.6°C, but was not subjected to pressure treatment.

After the requisite pressure and temperature treatments, all of the samples were diluted by factors of 10, 100, and 1000, spread on LPD plates (1% yeast extract, 2% peptone, 2% dextrose, 1.5% agar), and grown at 32°C for 24 hours. The number of colony forming units (cfu) were calculated by multiplying the number of colonies by the dilution factor.

A pronounced inactivation of *Saccharomyces cerevisiae* by pressure treatments was observed at both 23.8°C and -3.6°C. The colony forming activity of the pressurized sample ranged from 1 X 10^2 cfu to 5.6 X 10^3 cfu for the pressurized samples. The positive controls ranged in colony forming activity from 1.1 x 10^5 cfu to 9.2 x 10^5 cfu. Thus, the colony forming ability of *Saccharomyces cerevisiae* was decreased by approximately 2 to 3 orders of magnitude.

Example 5: Pressure and Temperature Induced Inactivation of the Moloney Murine Leukemia Virus

A retroviral vector, pLNCX, containing Phi+ (the extended viral packaging signal) and a neomycin resistance marker (Neo^r), but lacking viral structural genes, was used in conjunction with a packaging cell line, NIH-Cyt2. The NIH-Cyt2 cells express the gag, pol, and env viral structural genes necessary for particle formation and replication, but not the RNA packaging signal Phi+. Thus, the pLNCX vector and NIH-Cyt2 cell line together produce infectious, replication-incompetent particles structurally identical to the Moloney Murine Leukemia Virus (MMLV). These infectious MMLV particles contain RNA encoding the pLNCX vector.

MMLV infectious particles, suspended in DMEM with 10% calf serum (CS), are maintained at 4°C and 14.7 psi except during the pressure and temperature treatments described below. Hydrostatic pressure for one sample is cycled between 35,000 psi and 14.7 psi at 30 second intervals while the temperature is maintained at 2°C. As a positive control, the temperature of a second sample is maintained at 2°C for 10 minutes at 14.7 psi. A third sample is cycled between 35,000 psi and 14.7 psi at 30 second intervals at -10°C. A fourth sample is held at 35,000 psi for 5 minutes at -10°C. As a positive

control, a fifth sample is frozen in dry ice and then warmed to -10°C for 5 minutes.

Samples are added to dishes of NIH-Cyt2 cells and incubated at 37°C for 30 minutes to transfect cells with the retroviral vector pLNCX. As a negative control, one plate of cells is mock-transfected with viral-free DMEM-CS. The cells are grown in DMEM with 10% calf serum and G418 which only allows cells with Neo^r to grow and thus selects for stable transformants. After a 10 day period, the dishes are rinsed with PBS, stained with methylene blue, rinsed again with PBS, and then the colonies are counted. The viral titer, expressed in colony forming units (cfu), is calculated by multiplying the dilution factor by the number of colonies.

Example 6: Pressure-cycling inactivation of *Escherichia coli*-inoculated serum

A culture of ampicillin resistant *E. coli* strain in LB/amp media (Luria broth containing 50 μg/ml ampicillin) was grown to saturation. A sample of adult bovine serum (Sigma) was inoculated with 30 μl *E. coli* solution per ml serum. Aliquots (280 μl each) of the inoculated serum were placed into nine microcentrifuge tubes. The samples in the nine tubes were subjected to the following experimental conditions:

Sample 1 was left untreated as a control.

Sample 2 was maintained at atmospheric pressure (i.e, about 14.7 psi) as the temperature was cycled twenty times between about -17°C and ambient temperature (i.e., about 25°C) over 20 minutes.

Samples 3-6 were cooled to -15°C at atmospheric pressure. The samples sat for 2 minutes to ensure thermal equilibrium. The samples were then subjected to pressure cycles at a rate of about 30 sec/cycle, or 2 Hz. Sample 3 was cycled ten times between about 14.7 psi and about 15,000 psi over 5 minutes; sample 4 was cycled ten

times between about 14.7 psi and about 35,000 psi over 5 minutes; Samples 5 and 6 were cycled twenty times between about 14.7 and about 35,000 psi over 10 minutes.

Sample 7 was cycled twenty times between about 14.7 psi and about 35,000 psi at ambient temperature over 6 minutes, forty seconds.

Samples 8 and 9 were subjected to static pressurization for 2.5 minutes; sample 8 was pressurized at ambient temperature and sample 9 was pressurized at -15°C.

Dilutions of the samples were made in LB/amp media, and the samples were plated. Colony forming units per milliliter (CFU/ml) are shown below, along with log reductions (i.e., relative to the control):

Sample #	CFU/ml	Log reduction
1	1×10^6	0 (control)
2	3×10^5	0.5
3	$< 1 \times 10^6$	> 0
4	8×10^4	1.1
5	1×10^4	2.0
6	6.4×10^4	1.2
7	80	4.1
8	9.6×10^3	2.0
9	4×10^4	1.4

Thus, the most effective treatment was that corresponding to sample #7, cycling to 35,000 psi at about 25°C. The results indicate that cycling was more effective than static pressurization at the same temperature (cf. sample #8).

Example 7: Sterilization of *E. coli*-contaminated needles

Two 20G needles were clipped, leaving 3 mm of the metal shaft attached to the plastic mounts. Tubes were constructed by cutting the ends off the plastic shaft of a 3 ml syringe and plugging each end with the rubber

portions from two plungers. One ml of an overnight culture of *E. coli* was passed through each needle. Each needle was placed in a tube with 1 ml luria broth (LB) medium. There was approximately 0.2 ml of air left at the top of each tube.

One tube was subjected to 10 cycles of pressure treatment (each cycle including 30 seconds at 37,000 psi, followed by 30 seconds at 14.7 psi) at 22.2°C. The second tube was placed inside the pressure chamber for 10 minutes at 22.2°C but was not subjected to pressure treatment.

After pressure treatment, the needles were removed from the tubes and 0.2 ml LB was passed through each needle. Half of the 0.2 ml LB was spread on an LB plate. The remaining 0.1 ml LB was diluted (1:10, 1:100, 1:1,000, ad 1:10,000) and spread on four LB plates. All plates were grown overnight at 37°C. Colonies were counted on each plate and the number of colony forming units (cfu) within each LB sample was calculated.

A dramatic difference was observed in the colony forming activity of the LB medium passed through the treated and untreated needles. There were 10 cfu in the 0.2 ml passed through the pressure treated needle, while there was 9.2×10^5 cfu present in the LB passed through the untreated needle, nearly a 100,000-fold difference.

Example 8: Pressure-shock sterilization

As a model for pathogenic viruses, fresh frozen plasma is spiked with 10^8 plaque forming units (pfu) per ml of lambda bacteriophage stabilized with 10% glucose and 4 mM sodium caprylate. The plasma is placed in a high pressure vessel containing 50% ethylene glycol as a pressure transmitting medium and the temperature is equilibrated to -10°C. The pressure is increased to 150,000 psi over a period of 1 second and held at that pressure for an additional 1 second. The pressure is

then released over the course of 2 seconds. The plasma sample is removed and dilutions are plated on lawns of *E. coli*. The plasma sample is found to be substantially free of infectious virus. The plasma proteins are analyzed by various methods including HPLC, IgG antigen binding, fluorescence enhancement of dansyl sarcosine by HSA, and clotting assays (including the activated partial thromboplastin time assay, APTT) to assess the integrity of the clotting factors.

Example 9: Pressure-enhanced photochemical inactivation of viruses

A sample of bovine serum is inoculated with 10^8 plaque forming units (pfu) per ml of lambda bacteriophage. 0.15 mM of psoralen is added. The sample is split into three aliquots. The three samples are treated as follows:

(1) no further treatment
(2) pressurization to 30,000 psi
(3) pressurization to 30,000 psi and simultaneous exposure to UVA light for 10 minutes.

All samples are held at a temperature of 25°C throughout the experiment. Treatment with pressure and light is accomplished by loading the sample into a quartz bottle with a polyethylene cap. The bottle is placed in an ethanol-filled high pressure spectroscopy cell (ISS, Champaign, IL) and pressurized. Samples are illuminated by aligning a window of the spectroscopy cell with a UVA lamp at a fixed distance. After treatment, the serum is serially diluted, mixed with *E. coli* and plated on agar. After overnight incubation at 37°C, the plaques on the plates are counted to arrive at the relative reduction of viral titer due to pressurization and the combination of pressurization and illumination. It is found that a

significantly greater degree of viral inactivation is observed in sample #3, relative to samples #1 and #2. The experiment is repeated with lower concentrations of psoralen with the result that the combination of pressure and UVA light gives a rate of inactivation similar to that obtained by UVA alone, while resulting in less damage to therapeutic proteins. Similar experiments reveal that less light intensity or time of illumination is needed when the sample is pressurized. Experiments also show that nucleic-acid binding dyes that act in conjunction with oxygen (e.g. methylene blue) give results similar to those seen with psoralens.

Example 10: Pressure enhanced chemical inactivation of viruses

A sample of bovine serum is inoculated with 10^8 plaque forming units (pfu) per ml of lambda bacteriophage. The sample is split into four aliquots. The samples are treated as follows:

#1 no treatment

#2 0.1 mM iodine added and incubated for 10 minutes

#3 0.1 mM iodine added and pressurized to 30,000 psi for 10 minutes

#4 pressure of 30,000 psi for 10 minutes

All samples are held at a temperature of 25°C throughout the experiment. After treatment, the reaction is quenched with a reducing agent and the serum is serially diluted, mixed with *E. coli* and plated on agar. After overnight incubation at 37°C, the plaques on the plates are counted to arrive at the relative reduction of viral titer due to pressurization and the combination of pressurization and chemical treatment. It is found that sample #3 has significantly greater reduction in viral titer (as compared to sample #1) than the sum of the

reductions observed from samples #2 and #4, demonstrating a synergistic effect of pressure and iodine. Similar experiments are carried out with lower concentrations of chemical additives and it is found that pressure allows equivalent viral inactivation with lower concentrations of iodine or with shorter incubation time.

Example 11: Effects of pressure cycling treatment on clotting time

Human plasma samples (BBI) were treated with 10, 2-minute pressure cycles at 40,000 psi and temperatures ranging from -70°C to 20°C, and stored frozen at -70°C until use. The assay was initiated by addition of 60 µl plasma, 60 µl APTT reagent (Sigma) (37°C), and 60 µl of 25 mM $CaCl_2$ (37°C) to a 100 µl cuvette. The absorbance was observed at 550 nm and the clotting time was recorded as the time to an OD of 0.8 (approximately 1/2 maximum). It was found that the use of lower temperatures gave a dramatic improvement in clotting activity.

Example 12: Integrity of IgG, IgM, HSA, and fX in cryobaric treatment

IgG and IgM. Commercial preparations (ACCURUN™, Boston Biomedica Inc.) of human plasma controls for Cytomegalovirus (CMV) antibody titer were treated with the pressure cycling technique and the ability of IgG and IgM to bind CMV antigen was measured using Abbott and Walpole CMV -antibody enzyme immunoassays. The assay comprises incubating the sample with polystyrene beads which are coated with heat inactivated CMV antigen. The beads are then washed and reacted with horseradish peroxidase labeled anti-human immunoglobulin. The beads are washed again and incubated with a chromogenic substrate which develops color at a rate indicative of the bound anti-CMV antibody. Dilutions of the ACCURUN material indicate that the assay is linear, suggesting

that any loss of activity would have been detected. 260 μl samples of Accurun were equilibrated to -5°C and treated with 10 pressure cycles comprising 1 minute at elevated pressure and 1 minute at atmospheric pressure. Maximal pressures of about 10,000 to about 50,000 psi were used. Samples were removed and anti-CMV levels were measured. No significant loss of IgG or IgM activity was observed for pressures of up to about 50,000 psi.

HSA. Human serum albumin (HSA) integrity was determined by its ability to enhance the fluorescence of dansyl sarcosine. This assay is sensitive to changes in domain III of the protein and to overall levels of aggregation. Forty mg/ml of HSA was added to bovine serum which had been filtered with a microcon-30 spin-filter to remove any bovine albumin and larger proteins. The samples were subjected to pressure pulsation with a maximum pressure of about 34,000 psi for 15 minutes with various frequency of pulsation at -5°C. Protein activity was measured by successive addition of 10 μl volumes to a 3 ml solution of excess dansyl sarcosine in PBS. The results indicated that pressure pulsation is beneficial to protein integrity.

Factor X. To test the effect of the cryobaric process on an enzymatic blood factor, a chromogenic assay was performed on human ACD plasma treated at several pressures. The conditions used were identical to those in the IgG experiment: -5°C, 10 two-minutes cycles at about 19,000 psi to about 50,000 psi. Greater than 80% of factor X activity remained.

Conclusions:

This data demonstrates that pressure cycling is capable of greater than 10^7 reduction in titer of non-enveloped virus in a 15-minute period, yet IgG, IgM, factor X, and HSA activities can simultaneously be maintained at acceptable levels. Pulsation of pressure appears to enhance the retention of protein activity.

Example 13: Pressure cycling inactivation of porcine parvovirus (PPV) in human plasma at very low temperature

Porcine Parvovirus (ATCC) is propagated in porcine testicle (PT) cells by infecting at a multiplicity of infection of 0.1 and incubating for 6 days. The cells are lysed by three freeze-thaw cycles and the solution is clarified by centrifugation. The resultant PPV titer is approximately 1 x 10^{10} infectious doses/ml.

Stock PPV is diluted 1:100 into human plasma and dispensed in 260 μl volumes into two flexible polyethylene microcentrifuge tubes (Cole Parmer). One tube is designated "control" and the other "experimental". The tubes are sealed with Dow Corning silicone high vacuum grease, and wrapped in PARAFILM® (American National Can, Menasha, WI).

The high pressure reaction chamber is filled with 70% ethanol and 0.2% rhodamine B as a tracer and cooled to -40°C. The experimental tube is placed in the high pressure reaction chamber for 6 minutes to equilibrate the temperature and the pressure is increased over a period of 5 seconds to a pressure of 80,000 psi. The pressure is held for 10 minutes at 80,000 psi and then released over a period of 2 seconds. The temperature is then allowed to equilibrate for 5 minutes. This process is repeated for a total of 20 cycles. The control sample is incubated for an equivalent time at -10°C.

The PPV titers of the control and experimental samples are determined by performing half-log serial dilutions in Hanks Buffered Saline Solution (HBSS). Each dilution is used to inoculate 6 wells of a 96 well culture plate containing 50% confluent PT cells.

After incubating the cells for 5 days in MEM media supplemented with 7.5% fetal bovine serum, the presence of virus is determined by fixing the plates in 80% acetone for 20 minutes and incubating each well with 50

μl of fluorescein-conjugated anti-PPV antibody (VMRD Inc., Pullman WA) for 40 minutes at 37°C, washing 3 times with water, and observing with an epifluorescence microscope. TCID50 is determined by the Spearman-Karber method. The experimental sample is found to have no detectable virus and the control sample has a titer of 10^8 infectious doses/ml. Clotting time analysis is performed by a single stage activated partial thromboplastin time (APTT) measurement and the clotting time is found to be about 30 seconds for both the control and experimental samples indicating that the plasma is suitable for purification of blood products and transfusion.

Example 14: Pressure cycling inactivation of bacteria and fungi

Various species of bacteria were subjected to pressure-cycling protocols to determine the rate of inactivation. Two-hundred and fifty microliter volumes of each species were aliquoted into Cole Palmer polyethylene tubes. The tubes were frozen at -70°C and a layer of high vacuum grease was added to the top of the frozen liquid layer. Each tube was then wrapped in a layer of PARAFILM® (American National Can, Menasha, WI).

The following bacterial species were tested: *Bacillus cereus* ATCC 14579 (initial titer: 6.6×10^8 cfu/ml), *Enterococcus faecium* ATCC 49032 (3.3×10^8), two samples *Escherichia coli* ATCC 43894 (8.6×10^9 and 5.4×10^7), *Pseudomonas aeruginosa* ATCC 14502 at 9.5×10^7, and *Staphylococcus aureus* ATCC 9144 at 9.9×10^7.

Ten two-minute pressure cycles (i.e., 1 minute at ambient pressure, one minute at up to 50,000 psi) were carried out at -10°C on each bacterial culture. Certain samples of each species were also subjected to 10 minutes of static pressure at both about 29,000 and about 50,000 psi. Each sample was equilibrated to the chamber temperature for five minutes prior to pressurization.

The chamber was filled with 50% ethylene glycol in water and spiked with 1% rhodamine, to determine if any of the chamber liquid has leaked into the sample during pressurization. Following the pressure cycling procedure, the samples were placed at -70°C until assayed.

The samples were assayed to determine the titer (cfu/ml) following the pressurization process. Serial dilutions were made from an aliquot of each sample. Each dilution was plated onto 10 ml solid agar and incubated overnight at 37°C. The titer of each plate was counted to determine the rate of inactivation (final titer). The initial titer was divided by the final titer to determine the reduction in microbial number.

All gram positive and gram negative bacteria examined were inactivated. *Bacillus cereus* was inactivated by 5 logs, *Enterococcus faecium* by 6 logs, E. coli by 7 logs, *Pseudomonas aeruginosa* by 8 logs, *Staphylococcus aureus* by 6 logs and *Candida albicans* by 5 logs. For all bacteria, pressure pulsation was more effective than static pressure. The most dramatic difference was seen with *Enterococcus*, which was inactivated by less than 1 log without pressure cycling but by approximately 6 logs with pressure cycling at 50,000 psi. *Pseudomonas* was inactivated by approximately 6 logs with pressure cycling and 2.5 logs with static pressure at around 29,000 psi. The rate of *Pseudomonas* inactivation at 50,000 psi with pressure cycling was greater than 8 logs, but with static pressure remained at 6 logs. The *Staphylococcus* sample was inactivated by 2 and 6 logs with pressure-cycling at 29,000 psi and 50,000 psi, respectively, but only 1 and 3 logs with the same conditions under static pressure. The difference in the rate of inactivation of the low titer *E. coli* at 29,000 psi was 67-fold with cycling and 6.4-fold with static pressure. The rate of inactivation at 50,000 psi

increased to 4 logs with cycling but only to 2 logs with static pressure. The high titer *E. coli* yielded an increase of 4 logs of inactivation with pressure cycling compared to static pressure at both 29,000 psi and 50,000 psi.

Pressure pulsation thus yielded an increase in inactivation over static pressure for all species of bacteria tested, with the benefit of pressure-cycling being more pronounced at 50,000 psi than at 29,000 psi for all species.

Example 15: Inactivation of enveloped viruses

To test the efficacy of cryobaric treatment against enveloped viruses, Human Immunodeficiency Virus (HIV-1) and Herpes Simplex Virus (HSV-1) were treated and their infectivity measured. For the HIV experiment, the cryobaric treatment consisted of 5 two minute pressure cycles at -10°C. Each cycle consisted of 1 minute at high pressure and 1 minute at atmospheric pressure. An approximately 6 log reduction in HIV infectivity was achieved when the pressure was elevated to 50,000 psi. Herpes Simplex 1 was also inactivated by 6 logs at this pressure. This treatment is compatible with retention of the integrity and activity of most therapeutic proteins, as shown, for example, in Example 21.

Example 16: Inactivation of MS2: static pressure conditions

MS2-infected plasma samples were pressurized to 80,000 psi at -26°C, then the temperature was increased to -14°C for 5, 10, 15, 30 and 60 minutes. Two plasma samples were treated for each experiment, one with phage for inactivation studies and one without phage for factor analysis.

Following cryobaric treatment, MS2 infectivity was assayed according to the following protocol. Host *E.*

coli were grown to saturation overnight in MS2 Broth (containing 10 g/l tryptone and 42 mM sodium chloride), supplemented with 0.2% maltose and 10 mM magnesium sulfate. Following dilution of treated phage, where necessary, 100 µl of phage sample was added to 100 µl of the *E. coli*, incubated at 23°C for 20 minutes, then incubated at 37°C for 10 minutes. The phage-*E. coli* mixture was added to 2.5 ml melted Top Agar (10 g/l tryptone, 42 mM sodium chloride, 7 g/l agar, 47°C), vortexed, and immediately spread onto 90 mm petri dishes containing 10 g/l tryptone, 42 mM sodium chloride, and 10 g/l agar. After incubation for approximately 16 hours at 37°C, plaques were counted.

The samples for factor VIII analysis were stored at -70°C until assay. Factor VIII assays were performed with the American Diagnostica chromagenic assay kit (Greenwich, CT).

The results were as follows, with "Inactivation" representing the titer (in pfu/ml) for the untreated sample divided by the titer for each given sample, and "fVIII" representing the percentage of factor VIII activity remaining for each sample after pressure treatment (i.e., relative to the untreated sample):

Time(min)	Titer	Inactivation	fVIII
0	3.2e9	----	100% (defined)
5	7.5e6	4.3e2	95
10	7.0e6	4.6e2	79
15	5.4e6	5.9e2	87
30	6.0e6	5.3e2	72
60	1.3e6	2.6e3	67

These data show that approximately first order kinetics were observed for factor VIII loss, while MS2 inactivation was relatively independent of incubation

time, suggesting that the viral inactivation was occurring in the initial pulse to 80,000 psi at -26°C.

Example 17: Inactivation of MS2: temperature optimization

In an experiment to determine the optimum temperature for MS2 inactivation, the conditions indicated in Example 16 were employed with the exception that each sample was equilibrated for 5 minutes to the corresponding starting temperature indicated in the data table below. The samples were pressurized to approximately 80,000 psi for 2 minutes, followed by depressurization and temperature equilibration for 5 minutes. The pressurization cycle was repeated 2 more times. The results were as follows:

Temperature	Titer	Inactivation	fVIII
-20°C	3.2e6	3.4	47%
-17°C	4.6e5	4.2	36%
-14°C	1.2e4	5.8	9%
-11°C	5.0e3	6.2	0%
-11°C	7.4e9		100% (control)

These data demonstrate significant (i.e., 3 log) inactivation of MS2 under conditions (-20°C) that give 50% recovery of fVIII. The higher temperatures increased inactivation of MS2 but decreased recovery of fVIII.

Example 18: Inactivation of MS2: pressure optimization

In an experiment to determine the optimum pressure for MS2 inactivation, the conditions indicated in Example 16 were employed with the exceptions that each sample was equilibrated to -17°C for 5 minutes and the samples were pressurized to between 60,000 and 80,000 psi, as indicated below. The indicated pressures were held for 60 seconds, followed by depressurization and temperature equilibration for 5 minutes. The time of pressurization

(about 3 seconds) and depressurization (about 1 second) are not included in the reported pressurization time. The results were as follows:

Pressure (psi)	Titer	Log inactivation	fVIII	%loss/log
60,000	2.6e9	0.3	53	157
65,000	1.3e9	0.6	45	91
70,000	7.0e7	1.9	47	28
75,000	9.4e5	3.8	47	14
80,000	1.1e6	3.7	32	18
control	5.8e9			

The data indicate that the optimum pressure under these temperature and timing conditions was about 75,000 psi.

Example 19: Inactivation of MS2: pulsation timing optimization

To measure the relative rates of MS2 inactivation and loss of fVIII activity as a function of the time at elevated pressure for each pulse, conditions similar to those employed in Example 18 were used. Each sample was equilibrated to -20°C for 5 minutes. The samples were pressurized to approximately 80,000 psi and held there for between 15 and 120 seconds, as indicated below, followed by depressurization and temperature equilibration for 5 minutes. The pressurization cycle was repeated 2 more times. The times required for pressurization (i.e., about 3 seconds) and depressurization (i.e., about 1 second) are not included in the reported pressurization time. A second round of experiments was conducted with pressure pulse durations of 10 to 120 seconds. The results of the two experiments were as follows:

Time (sec)	Titer	log inactivation	fVIII	loss/log

15	6.4e5	4.0	73	6.5
30	7.2e6	2.9	65	12
60	1.2e6	3.7	48	14
120	3.0e6	3.3	51	15
240	1.5e6	3.6	44	15
control	5.8e9			
10		3.3	72	8.5
15		3.7	65	9.4
30		4.0	44	14.0
120		4.8	19	16.9

These data indicate that decreasing the pressure pulse duration to times as short as 10 seconds has a positive effect on the retention of fVIII while having little or no effect on the level of MS2 inactivation.

Example 20: Inactivation of MS2: pH effect

Three milliliters of human plasma was spiked with 50 µl of MS2 stock in LB. A 10X stock solution of 3-[cyclohexylamino]-1-propane sulfonic acid (CAPS) was prepared and diluted 1:10 with spiked plasma. This sample and a plasma-only control sample were frozen at -70°C and sealed with silicone grease and PARAFILM®. Before treatment, each sample was placed into the reaction chamber for 4 minutes to equilibrate to the experimental temperature.

100 µl of each sample was removed for serial dilution. Phage were 100-fold serially diluted in lambda broth in microcentrifuge tubes. To ensure proper mixing, the tubes were vortexed before further dilution. 100 µl of each dilution was mixed with 100 µl of log phase MS2-host *E. coli*, incubated for 10 minutes at room temperature, and incubated for 20 minutes at 37°C. Three milliliters of lambda top agar (at 47-50°C) was added, then the tubes were briefly vortexed and poured over

lambda agar plates. The samples were exposed to a pressure of 60,000 psi for 10 minutes, then assayed.

The results were as follows:

Condition	titer
100 mM CAPS pH 10.5	3.0e6
control	3.9e9

The data indicate that addition of buffer to increase the pH allowed inactivation of the virus to occur at a pressure at which no activation occurred upon treatment of the neutral solution.

Example 21: Inactivation of pathogens in clinical specimens Samples of human plasma were equilibrated over a period of 4 minutes to -10°C. The pressure was increased to an elevated pressure of 20,000 psi for 1 minute. The pressure was reduced to atmospheric pressure and equilibrated for 1 minute. This process was repeated nine more times. Similar treatments were performed using elevated pressures of 30,000, 40,000, 50,000 and 60,000 psi. A control sample was held at -10°C for an equivalent period of time (about 20 minutes), without increasing the pressure.

After treatment, clinical assays were performed to determine the activity of various analytes. The following analytes were unaffected by any of the pressure cycling treatments:

Uric acid
Calcium
Phosphorous
Glucose
Creatinine
Urea Nitrogen
Na, K, Cl, Mg, Bicarbonate
Triglycerides GB
Total Bilirubin
Total Protein
Albumin
Cholesterol

Lipase
Anion Gap
BUN/Creatinine Ratio
Albumin/Globulin Ratio
Alkaline phosphatase

The activities of the following multimeric enzymes were reduced in the samples exposed to pressures of 30,000 psi or more:

Creatine kinase (CK)
Alanine aminotransferase (ALT)
Aspartate aminotransferase (AST)
Liver alcohol dehydrogenase (LD)
Gamma glutamyl transferase (GGT)

Although there was enough residual activity remaining, for example, to measure such differences between normal and elevated levels of these enzymes as would occur in a patient with liver disfunction, a sterilization method that does not reduce the activity of multimeric enzymes was desired.

To this end, the following experiment was carried out: Human plasma samples were equilibrated to -10°C for 4 minutes and the pressure was elevated to 80,000 psi over a period of about 5 seconds and held for a defined duration. The pressure was then lowered to ambient over about 2 seconds. The process was repeated 2 more times. The durations at elevated pressure tested were 1, 5, 10, 30, 60, 120, and 240 seconds. The plasma samples were then tested for enzymatic activity.

The activity of the two monomeric enzymes tested, lipase and amylase, were unaffected at any pressure duration.

With a 30 second or greater duration, the activities of LD, CK, AST, and ALT were almost completely eliminated while the activities of GGT and alkaline phosphatase (ALK) were reduced by about half. With a 5 second duration, all of the enzyme activities were improved. With a 1 second duration, ALT, GGT and ALK had near

complete activity, and LD, AST and CK had 40% to 70% of the activity of the untreated control.

Even at the short durations, acceptable levels of inactivation of pathogens can be obtained, for example, in the preceding Examples.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A method for sterilizing a material containing a microbe, the method comprising:

providing a material at an initial pressure and temperature;

increasing the pressure to an elevated pressure sufficient to inactivate at least some of the microbe in the material; and

decreasing the pressure to a reduced pressure, thereby providing a sterilized material.

2. The method of claim 1, wherein the material further comprises a protein, and the elevated pressure is insufficient to irreversibly denature the protein.

3. The method of claim 1, wherein the elevated pressure is sufficient to inactivate at least about 10% of the microbe in the material.

4. The method of claim 1, wherein the reduced pressure is about 1 atm.

5. The method of claim 1, wherein the initial temperature is about 25°C.

6. The method of claim 1, wherein the initial temperature is less than about 0°C.

7. The method of claim 6, wherein the initial temperature is between -20°C and -5°C.

8. The method of claim 1, wherein the elevated pressure is in the range of about 5,000 psi to about 95,000 psi.

9. The method of claim 1, wherein the elevated pressure is in the range of about 30,000 to about 75,000 psi.

10. The method of claim 1, wherein the elevated pressure is in the range of about 95,000 to about 150,000 psi.

11. The method of claim 1, further comprising repeatedly cycling the pressure between the elevated pressure and the reduced pressure.

12. The method of claim 11, further comprising cooling the material to a decreased temperature prior to the pressure increasing and cycling steps.

13. The method of claim 12, wherein said decreased temperature is between about -40° and about 0°C.

14. The method of claim 12, wherein the decreased temperature is between about -20° and about -5°C.

15. A method of producing a vaccine against a pathogen, the method comprising:

obtaining a suspension of pathogenic cells;

sterilizing the suspension by the method of claim 11; and

adding an adjuvant to the sterilized suspension to produce a vaccine.

16. The method of claim 15, further comprising, after the sterilizing step, removing from the suspension substantially all of any toxins that may be present in the suspension.

17. The method of claim 11, wherein the initial temperature is between -40°C and 0°C.

18. The method of claim 11, further comprising providing sufficient time to allow the temperature of the material to equilibrate prior to each increasing of the pressure.

19. The method of claim 1, further comprising cooling the material.

20. The method of claim 19, wherein the cooling step is carried out prior to the pressure increasing step.

21. The method of claim 19, wherein the cooling step is carried out after the pressure increasing step but before the pressure decreasing step.

22. The method of claim 19, wherein the material is cooled to a temperature in the range from about -40° to about 0°C.

23. The method of claim 19, wherein the material is cooled to a temperature in the range from about -20° to about -5°C.

24. The method of claim 19, further comprising warming the material to an increased temperature before the pressure decreasing step.

25. The method of claim 19, further comprising warming the material to an increased temperature after the pressure decreasing step.

26. The method of claim 1, further comprising, prior to the pressure increasing step, adjusting the pH of the material to a pH greater than about 10.

27. The method of claim 1, further comprising, prior to the pressure increasing step, adjusting the pH of the material to a pH less than about 4.

28. The method of claim 1, wherein the material being sterilized is selected from the group consisting of a biological sample, blood plasma, therapeutic and diagnostic products derived from blood plasma, biological fluids, medical fluids, medicaments, research solutions and reagents, serum, living tissue, medical or military equipment, a foodstuff, a pharmaceutical preparation, and a vaccine.

29. The method of claim 1, wherein the microbe comprises one or more members of the group consisting of a bacterium, a virus, a fungus, a protist, a spore former, protozoan parasites, helminth parasites, malaria-inducing organisms, giardia, and a virally infected cell.

30. The method of claim 1, wherein a phase-change catalyst is added to the material prior to sterilization.

31. A material sterilized by the method of claim 1.

32. The method of claim 1, wherein a protein-stabilizing agent is added to the material prior to sterilization.

33. The method of claim 32, wherein the protein-stabilizing agent is selected from the group consisting of sugars, glycerol, a hydrophilic polymer, a cyclodextrin, a caprylate, acetyl tryptophanoate,

polyethylene glycol, anti-oxidant, and a protein specific ligand.

34. The method of claim 1, wherein a nucleic acid-binding compound is added to the material prior to sterilization.

35. The method of claim 34, wherein the nucleic acid-binding compound is a photosensitizer.

36. The method of claim 35, wherein the photosensitizer is a psoralen.

37. The method of claim 1, wherein the material to be sterilized is provided in its final packaging, the packaging being adapted to transmit pressure without rupture.

38. The method of claim 37, wherein the packaging is hermetically sealed in flexible plastic.

39. The method of claim 37, wherein the packaging is a syringe and pressure is transmitted via a plunger.

40. A method for pressurizing an infectious material, the method comprising:

charging the material into a container adapted to transmit an external pressure to the material;

submerging the container in a sterilizing chemical solution; and

pressurizing the material within the container.

41. The method of claim 40, wherein the chemical sterilizing solution is selected from the group consisting of an oxidizing agent, an alcohol, urea, a guanidinium salt, an acid, and a base.

42. A method for inactivating a virus in a material, the method comprising:

(a) providing a material at an initial pressure and temperature; and

(b) exposing the material to repeated pressure cycles, each cycle comprising:

(i) increasing the pressure to an elevated pressure,

(ii) maintaining an elevated pressure for a time period t_e,

(iii) decreasing pressure to a reduced pressure, and

(iv) maintaining the material at a reduced pressure for a time period t_i,

wherein the elevated pressure is sufficient such that each cycle inactivates at least some of the virus in the material when the elevated pressure is maintained for time t_e.

43. The method of claim 42, wherein

the material includes a protein, and

the elevated pressure is sufficient to irreversibly denature substantially all of the protein if the elevated pressure is maintained for a time substantially longer than t_e, but wherein the elevated pressure is insufficient to irreversibly denature the protein when the elevated pressure is maintained for time period t_e or less.

44. The method of claim 43, wherein the elevated pressure is sufficient to denature substantially all of the protein if the elevated pressure is maintained for a time period longer than ten times t_e.

45. The method of claim 43, wherein the elevated pressure is sufficient to denature substantially all of

the protein if the elevated pressure is maintained for a time period longer than three times t_e.

46. The method of claim 43, wherein the protein comprises one or more blood clotting factors.

47. The method of claim 43, wherein the protein comprises one or more immunoglobulins.

48. The method of claim 43, wherein the protein comprises one or more monomeric proteins.

49. The method of claim 43, wherein the protein comprises one or more multimeric proteins.

50. The method of claim 43, wherein the material is selected from the group consisting of blood plasma, therapeutic and diagnostic products derived from blood plasma, biological fluids, medical fluids, medicaments, research solutions, living tissue, and pharmaceutical preparations.

51. The method of claim 42, wherein the virus comprises a non-encapsulated virus.

52. The method of claim 42, wherein the virus is selected from the group consisting of human parvovirus B19, porcine parvovirus, bovine parvovirus, human immunodeficiency virus, herpes simplex virus, hepatitis A virus, and transfusion transmitted virus.

53. The method of claim 42, wherein the elevated pressure is 10,000 to 120,000 psi.

54. The method of claim 42, wherein the elevated pressure is 40,000 to 100,000 psi.

55. The method of claim 42, wherein the elevated pressure is 70,000 psi to 90,000 psi.

56. The method of claim 42, wherein the reduced pressure is intermediate between the initial pressure and the elevated pressure.

57. The method of claim 42, wherein time t_e is 0.5 to 300 seconds.

58. The method of claim 57, wherein time t_e is 10 to 30 seconds.

59. The method of claim 42, wherein the material is exposed to between 2 and 100 cycles.

60. The method of claim 42, wherein the material is exposed to at least about 3 cycles.

61. The method of claim 42, wherein the material is exposed to at least about 10 cycles.

62. The method of claim 42, wherein the material is exposed to at least about 100 cycles.

63. The method of claim 42, further comprising, before the exposing step, cooling the material to a temperature T_e.

64. The method of claim 63, wherein T_e is -40°C to 10°C.

65. The method of claim 64, wherein T_e is -25°C to -10°C.

66. A sterilized material produced by the method of claim 42.

67. A method of producing a vaccine, the method comprising:

(a) obtaining a suspension of a virus;

(b) inactivating the virus by the method of claim 42; and

(c) adding an adjuvant to the suspension containing the inactivated virus to produce a vaccine.

68. A vaccine produced by the method of claim 67.

69. An apparatus for sterilizing a material, the apparatus comprising:

a pressurization vessel adapted to transmit an external pressure to a material within the vessel, wherein:

the vessel is capable of withstanding an elevated pressure;

the vessel is adapted to fit into a pressure cycling apparatus; and

the vessel includes a valve that allows aseptic recovery of the sterilized material.

70. The apparatus of claim 69, further comprising a heater and a refrigerator, wherein the heater and the refrigerator control temperature within the pressurization vessel.

IN THE UNITED STATES RECEIVING OFFICE

Applicant : BBI BioSeq, Inc.
Serial No. : PCT/US99/13461
Filed : 15 June 1999
Title : RAPID CRYOBARIC STERILIZATION AND VACCINE PREPARATION

Box PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

PETITION UNDER 37 CFR 1.182

Applicant hereby petitions the Commissioner to explicitly recognize what the U.S. PTO has already implicitly recognized: i.e., that Applicant adequately indicated both its intent to claim priority and the identity of the applications from which it claimed priority in the PCT/RO/101 filed in this case (Exhibit A). Therefore, Applicant petitions for an explicit acknowledgement of Applicant's priority claim in the above-captioned international application.

Applicant may be seriously prejudiced in prosecuting national phase applications without this relief, and no party or interest will be unfairly prejudiced if the relief is granted. Moreover, no other jurisdiction can grant this relief.

Summary.

On September 27, 1999, and October 12, 1999, Applicant was given reason to believe that the priority claims had been accepted when the International Bureau sent Applicant forms PCT/IB/304 (Exhibits C and D) indicating receipt of the priority documents transmitted from the US Receiving Office to WIPO.

Then, on November 24, 1999, the US Receiving Office apparently then changed its mind and mailed a PCT/RO/111 (Exhibit E) indicating that the priority claims were not recognized. By that time, it was already more than 17 months after the priority date and therefore too late to obtain acceptance of the priority claim, as Applicant discovered when its Petition For Correction of Obvious Error Under Rule 91(B) and (C) (Exhibit F) was denied as untimely.

recognizing the priority claims in this case. Applicant has already been advised by WIPO and certain national jurisdictions that the issue is one for the U.S. Receiving Office to decide, yet the U.S. Receiving Office now says it is too late to provide any relief. With the record in its current state, Applicant is at risk for loss of its priority claim in one or more foreign jurisdictions. As detailed below, such a result is manifestly unfair.

Background

Applicant filed a PCT Request (Form PCT/RO/101; "Exhibit A") with the U.S. Receiving Office on June 15, 1999, claiming priority from two U.S. patent applications filed on June 15, 1998 and October 2, 1998. At Sheet No. 4 of the Request, Applicant stated that the application is a "Continuation of USSN 09/097,852 filed 15 June 1998 and USSN 09/165,829 filed 2 October 1998." At Sheet No. 5 of the Request, in Box No. VI ("Priority Claim"), Applicant repeated the filing dates and serial numbers of the priority applications as items (1) and (2). The cell labeled "national application: country" was not filled out. However, Applicant checked the box (also within Box No. VI) requesting that the Receiving Office prepare and transmit to the International Bureau a certified copy of each of the earlier applications identified in the box as items (1) and (2). Since this option is available "only if the earlier application was filed with the Office which for the purposes of the present international application is the Receiving Office," and the United States Patent Office was the Receiving Office, it was clear that the priority applications listed in Box No. VI were U.S. applications. Thus, there is no question on the face of the PCT demand (Exhibit A) about the nationality, serial numbers and identities of the priority applications. Indeed, both the U.S. Receiving Office and the International Bureau were clear on this point, and the Receiving Office sent the proper U.S. priority applications to the International Bureau.

On July 26, 1999, Applicant received a Notification Concerning Payment of Prescribed Fees (Form PCT/RO/102), a Notification of the International Application Number and of the International Filing Date (PCT/RO/105), a Invitation to Correct Defects in the International Application (PCT/RO/106), and an Invitation to Correct Priority Claim (Form PCT/RO/110), each mailed on July 21, 1999. Applicant responded

Application, but did not respond to the Invitation to Correct Priority Claim. Applicant then received two Notifications Concerning Submission or Transmittal of Priority Document (Forms PCT/IB/304; "Exhibits C and D") mailed by the International Bureau of WIPO on September 20, 1999 and September 24, 1999, which indicated receipt of the two priority applications on September 17, 1999 and September 20, 1999, respectively. Having been notified that the U.S. Receiving Office had actually transmitted the priority applications to WIPO, Applicant fairly concluded that the U.S. Receiving Office had acknowledged the priority request. Nonetheless, the Receiving Office, on November 24, 1999, mailed a Notification Relating to Priority Claim (Form PCT/RO/111; "Exhibit E"), which indicated that,

> "[t]he priority claim is considered not to have been made because the applicant failed to respond to the invitation under Rule 26bis.2(a) (Form PCT/RO/110) within the prescribed time limit."

In response, Applicant filed a Petition for Correction of Obvious Error under Rule 91 (B) and (C) ("Exhibit F"). Applicant has been informed that Petitions under Rule 91 cannot be considered later than 17 months from the earliest priority date, which would have been November 15, 1999. The petition therefore stands denied.

Thus, the PTO gave applicant reason to think that the priority claim was clearly and adequately made, and Applicant could not have anticipated that the PTO would mail notification to the contrary on November 24, 1999, more than a week after the expiry of the period allowed for correction via Petition under Rule 91. Applicant now petitions the Commissioner to acknowledge the priority claim in this exceptional situation.

Applicant has been informed that WIPO will also not consider the priority claim because (in Exhibit F) the U.S. Receiving Office considers the priority claims not to have been made ("Exhibit G"). Similarly, national patent offices may refuse to deal with this issue, believing it to be entirely within the jurisdiction of the U.S. Receiving Office. Consequently, Applicant's only recourse for obtaining its priority claims is through this petition.

Filed herewith is a check for $130 in payment of the petition fee set forth in §1.17(h). Please apply any charges or credits to our Deposit Account No. 06-1050.

Respectfully submitted,

Date: 1/11/00

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In re Application of
BBI BIOSEQ. INC.
PCT No.: PCT/US99/13461
Int. Filing Date: 15 JUNE 1999
Attorney's Docket No.:07985/024W01
For: RAPID CRYOBARIC STERILIZATION AND VACCINE PREPARATION

: DECISION ON
:
: PETITION UNDER
:
: 37 CFR 1.182
:
:

This correspondence is in response to the petition submitted on 12 January 2000 under 37 CFR 1.182. The appropriate fee set forth in §1.17(h) has been charged to Deposit Account No. 06-1050 as authorized.

The petition requests a change to Applicants' priority claims in the above-captioned international application.

DISCUSSION

As mentioned in the petition decision of 08 February 2000, the PTO mailed an invitation on 21 July 1999 (FORM PCT/RO/110) to Petitioner to correct the priority claims, and this form specifically stated to the applicants the consequence of failure to respond within the time specified under PCT Rule 26*bis*.2(b). The consequence, briefly stated, "that for the purpose of the Treaty the priority claim shall be considered not to have been made." Accordingly, petitioner was informed and aware of the consequence of not responding to the Invitation mailed on 21 July 1999 in a timely manner.

The Receiving Office Acknowledges the Priority Country was indicated in the portion at Box 5 and possibly in the portion of Box 6, applicant requesting to transmit the priority documents to the International Bureau. Petitioner, however, mistakenly inferred this to satisfy the requirement to name the country of filing in the priority claim portion.

PCT Rule 4.10(Priority Claim) specifically states that "a statement to the effect that the priority of an earlier application is claimed and shall indicate:

(a)(iii) where the earlier application is a national application, the country party to the Paris Convention for the Protection of Industrial Property in which it was filed." In this case, the priority claim section of the Request at roman numeral box VI in the national application country portion, the indication was blank (was missing the application country). Therewithin lies the problem since the Receiving Office may not waive PCT Rule 4.10. Accordingly, it is inappropriate to grant relief beyond the acknowledgment of the indication of said portions.

Petitioner asserts, that "the PTO gave applicant reason to think that the priority claims were clearly and adequately made" because the International Bureau (IB) sent Applicants forms PCT/IB/304 indicating the receipt of the priority documents transmitted from the US Receiving Office to WIPO. The international bureau's notification was: 1) not an action by the PTO and 2) not a basis to conclude that the priority claims were in compliance with PCT Rule 4.10. The IB's notice merely conveyed receipt of the priority documents and not whether the priority claims were properly asserted.

Further, the PTO's 21 July 1999 notice, Invitation to correct the priority claims (Form PCT/RO/110), clearly stated the priority claims were not in accordance with PCT Rule 4.10 and the notice specifically set a one month time period for response. The required response to the PTO was due by 21 August 1999. Accordingly, it is unclear how Petitioner could have thought that the PTO "gave reason to think that the priority claims were made," when Petitioner received the communication (PCT/IB/304) from the IB on 27 September 1999 over a month after Petitioner was required to reply to PTO's notice (Invitation to correct priority claims).

Petitioner, nevertheless, further argues that " the US Receiving Office apparently then changed its mind when it mailed PCT/RO/111 (Exhibit E) indicating that the priority claims were not recognized." The Notification (mailed 24 November 1999) was not an indication that "the US Receiving Office apparently changed its mind" rather it was a clear statement that the consequences notified to applicants in the invitation were applicable because applicants failed to take timely corrective action.

Moreover, the PTO was very clear that it had recognized that priority claims were intended for the above international application, but the priority claims were incorrectly claimed since they did not satisfied the required indication set forth in Rule 4.10(a). PTO conveyed this to Petitioner on 21 July 1999 in an Invitation to correct the priority claims (Form PCT/RO/110). PCT Rule 26*bis*.2 is unambiguous and it clearly states that "[i]f, in response to an invitation under paragraph (a), the applicant does not, before the expiration of the time limit under Rule 26*bis* 1.(a), submit a notice correcting the priority claim so as to comply with the requirements of Rule 4.10, that priority claim shall, for the purposes of the procedure under the Treaty, be considered not to have made ,..." Based on the Invitation and Rule 26*bis*.2, it is clear Petitioner could not have thought otherwise especially when the Invitation was mailed in a timely manner affording petitioner ample time to either correct or to inquire about the priority claims of the above international application.

Petitioner appears not to have recognized that the priority documents and priority claims are two distinct, unrelated matters covered by different PCT rules. PCT Rule 17 deals with the priority documents being transmitted to the International Bureau. In this instance, transmitting the priority documents to the International Bureau as requested by petitioner on 15 June 1999 (PCT/RO/101) was proper under PCT Rule 17.1(b). PCT Rule 26*bis*.2, on the other hand, deals with invitations to correct defects in priority claims within a specified time period based on the priority date.

Petitioner's subsequent petition for correction of the priority claims was outside the time period allocated by PCT Rule 26*bis*.2 as it was discussed in the petition decision of 08 February 2000. After the expiration of the 26bis.2 time period, the US Receiving Office has no authority to grant relief to petitioner.

Notwithstanding the aforementioned, petitioner may still seek relief at the various national and regional offices.

CONCLUSION

For reasons above, the petition under 37 CFR 1.182 is **DISMISSED**, without prejudice.

Any further correspondence with respect to this matter may be directed to the Assistant Commissioner for Patents, Box PCT, Washington, D.C. 20231, and address the contents of the letter to the attention of the PCT Legal Office.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13461

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61L 2/00
US CL :422/39
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/39, 1, 28, 33, 292, 295, 307, 308

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS
search terms: pressure, sterilization, temperature, cyclic, pulsatile

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 3,445,568 A (ALLEN) 20 May 1969, whole document, especially columns 1-2.	1-70
Y	US 4,164,538 A (YOUNG et al) 14 August 1979, whole document, especially claims and figures.	1-70
Y	US 5,316,745 A (TING et al) 31 May 1994, whole document, especially claims.	1-70
Y	HAYAKAWA et al. Oscillatory Compared with Continuous High Pressure Sterilization on Bacillus stearothermophilus Spores, Journal of Food Science. 1994. Vol. 56. No. 1. pages 164-167, especially page 166.	1-70

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
30 AUGUST 1999

Date of mailing of the international search report
26 NOV 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT

Authorized officer
FARIBORZ MOAZZAM

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13461

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HASHIZUME et al. Kinetic Analysis of Yeast Inactivation by High Pressure Treatment at Low Temperatures, Biosci. Biotech. Biochem. 1995. Vol. 59. No. 8. pages 1455-1458, especially page 1457.	1-70